REMARKS

Status of the Claims

Claims 9, 11-16, and 22-24 are pending. Claims 9, 11, and 16 are amended. Claims 22-24 are newly added. Support for the amendments can be found throughout the application as originally filed. *See, e.g.*, Specification, ¶ 98. No new matter is added.

Claim 10 is cancelled without prejudice or disclaimer to the subject matter therein. Applicant reserves the right to pursue the cancelled subject matter in one or more divisional and/or continuation applications.

Claim Objections

Claims 9 and 16 are objected to for informalities.[1]

The Examiner suggests reciting expression of the DNA molecule in part (III) of claim 9 and inserting a comparison to a plant that does not comprise the DNA molecule.[2] Claim 9 has been amended to recite "wherein the DNA molecule, when introduced and expressed in an Arabidopsis, Brassica or tobacco plant, is capable of producing a plant tolerant to high light stress as compared to an Arabidopsis, Brassica, or tobacco plant that does not comprise said isolated DNA molecule."

As suggested by the Examiner, Claim 16 has been amended to insert a space between "(iii)" and "a." Claim 16 has also been amended to insert "as compared to an Arabidopsis, Brassica, or tobacco plant that does not comprise said chimeric gene."

In view of the foregoing, Applicant respectfully submits that the objections to claim 9 and 16 are now rendered moot.

Rejections Under 35 USC § 112

Claims 9-16 stand rejected under 35 U.S.C. 112, first paragraph, for lack of enablement.[3] Specifically, the Office Action states:

> [T]he specification, **while being enabling for a DNA molecule comprising a plant expressible promoter operably linked to an [sic] 163 bp of Par G coding sequence of SEQ ID NO: 3 (positions 973 to 1135) in sense and antisense orientation separated by an intron, and further operably linked to 3' transcription termination signals, to produce an inhibitory double-stranded RNA molecule when expressed in transgenic *Arabidopsis*,**

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[1] *See* Office Action, p. 3.

[2] *Id.*

[3] *Id.*

> ***Brassica*** **or tobacco plant, and wherein said transgenic plant exhibits high light stress tolerance, or a method of producing said transgenic plant using said DNA molecule,** does not reasonably provide enablement for a DNA molecule comprising at least 40, 50, or 100 nucleotides in sense and antisense orientation from any region of a nucleotide sequence encoding the protein of SEQ ID NO: 1.[4]

Applicant has amended claims 9 and 16 to recite sense and antisense nucleotide sequences of at least **163** consecutive nucleotides of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No. 1 or the nucleotide sequence of SEQ ID No. 3. Dependent claims 22 and 23 have been added, further specifying that the 163 consecutive nucleotides correspond to nucleotides 973-1135 of SEQ ID NO: 3. Applicant has also amended claim 9 to require that the DNA molecule be capable of producing a plant tolerant to high light stress when introduced into an Arabidopsis, Brassica or tobacco plant.[5] Claim 16 has been amended to recite that tolerance to high light conditions be compared to Arabidopsis, Brassica, or tobacco plants that do not comprise the recited gene. Accordingly, claims 9 and 16 now recite substantially all of the elements that the Office Action expressly admits are enabled.[6]

To the extent that the enablement rejection is based upon the selection of the region of the ParG gene to be encoded by the ParG inhibitory RNA molecule (i.e., from nucleotide X to nucleotide Y of the ParG gene), Applicant respectfully submits that one of skill in the art would have readily been able to design inhibitory RNA molecules encoded by multiple, different regions of the ParG gene. Applicant submits that it was known that the selected region of the target gene coding region is not critical when designing long hairpin RNA silencing constructs. For example, in a 2005 article ("the De Block article"), the down-regulation of PARP activity was achieved using inhibitory RNA encoded by multiple, different regions of the PARP gene.[7] In the De Block article, several different silencing constructs are described, including two designated hp*ATParp2* and hp*ATParp2(signature)*.[8] The hp*ATParp2* construct encodes a dsRNA region targeted towards the N-terminal region of the *AtParp2* gene, including nucleotides 189-

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[4] *Id.* at pp. 3-4.

[5] It is understood that the DNA molecule is also capable of producing a plant with increased tolerance to high light stress when introduced into other species.

[6] *See id.*

[7] De Block et al., Plant Journal 2005, 41, 95-106 (Attached as Exhibit A).

[8] *Id.* at p. 103 (1st column, "Experimental Procedures—Plasmid Constructions").

781 of *AtParp2*.[9] The *hpATParp2(signature)* construct, on the other hand, encodes a dsRNA region targeted towards the C-terminal region of the *AtParp2* gene, including nucleotides 1572-1730 of *AtParp2*.[10] Both of these constructs down-regulate the activity encoded by the *AtParp2* gene to improve the stress tolerance of the modified plants.[11] Accordingly, one of skill in the art would have understood that different regions of a gene could be used to design an inhibitory RNA construct.

Applicant also submits that one of skill in the art, in view of the specification, would have been able to identify the appropriate region or regions without undue experimentation.[12] The specification teaches one of skill in the art how to design inhibitory RNA molecules and introduce them into plant cells.[13] The specification also teaches one of skill in the art to identify plant lines in which the ParG gene is effectively silenced "by subjecting the plant lines to a particular adverse condition, such as high light intensity, oxidative stress, drought, heat etc. and selecting those plants which perform satisfactory [*sic*] and survive best the treatment."[14] Accordingly, it would have required merely routine experimentation to determine which inhibitory RNA constructs based on the ParG coding region were effective at inhibiting ParG and promoting stress tolerance.[15]

Finally, Applicant respectfully submits that the Office Action's citation to Arziman is not relevant to the enablement inquiry.[16] Applicants acknowledge that off-target effects may indeed occur. This is irrelevant, however, because the present application merely claims a plant tolerant to high light stress upon introduction and expression of a ParG inhibitory molecule, regardless of possible off-target effects.

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[9] *Id.*

[10] *Id.*

[11] *See id.* at pp. 97-98.

[12] *See In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988).

[13] *See, e.g.*, Specification, ¶¶ [27]-[56].

[14] *See id.* at ¶ [49].

[15] *See In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988).

[16] *See* Office Action, pp. 4-5 (citing Arziman for the proposition that off-target effects may occur if siRNAs have sequence homology to genes that are not supposed to be targeted).

For the foregoing reasons, Applicant submits that one of skill in the art would have been able to perform the claimed invention without undue experimentation. Accordingly, Applicant respectfully requests withdrawal of the enablement rejection.

Rejections Under 35 USC § 103

Claims 9-16 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Chang et al. (WO03000898), in view of Wesley et al. (2001) and Panda et al (2002).[17] Applicant respectfully traverses this rejection.

A. The Claims

The claims relate to DNA molecules encoding double-stranded inhibitory RNA molecules that down-regulate the activity of the ParG gene and plant cells and plants comprising this DNA molecule that possess increased tolerance to high light stress. Claims are also directed to methods of making Arabidopsis, Brassica, or tobacco plants tolerant to high light stress comprising the creation of transgenic plants with the DNA molecule and the selection of plants that exhibit the desired tolerance to high light stress.

B. The Office Action

The Office Action alleges that Chang teaches the down-regulation of the ParG gene of SEQ ID NO: 3 in Arabidopsis and Brassica plants by the transformation of a plant with a DNA construct whose transcription product inhibits ParG expression.[18] The Office Action expressly acknowledges that Chang does not teach using RNAi (double stranded inhibitory RNA)-based methods of down-regulating endogenous plant gene expression.[19] The Office Action also implicitly admits that Chang fails to disclose that down-regulation of the ParG gene confers increased tolerance to high light stress.[20]

To remedy the deficiencies of Chang, the Office Action cites Wesley as generally teaching hpRNA-based methods of gene silencing in plants (i.e. RNAi).[21] The Office Action

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[17] *Id.* at pp. 6-7.

[18] *Id.* at p. 7 ("Chang...teach[es]...a ParG (poly(ADP-ribose) glycohydrolase) nucleotide sequence as defined in SEQ ID NO: 550 *which is identical in sequence* to instant SEQ ID NO: 3.") (emphasis added).

[19] *Id.* at p. 8.

[20] *See id.* (discussing how the Panda reference allegedly teaches improved tolerance to high light stress in plants with down-regulated ParG activity).

[21] *Id.*

also cites Panda as allegedly teaching Arabidopsis mutants with disrupted ParG expression that are tolerant to high light stress.[22]

C. The Office Action Mischaracterizes Chang and Panda

Applicant respectfully disagrees with the Office Action's characterization of Chang. As an initial matter, Chang does not specifically teach a molecule that inhibits the expression of endogenous ParG expression, as alleged in the Office Action.[23] Rather, Chang provides a generic teaching of methods of suppressing gene activity, none of which are the double-stranded RNAi gene suppression technology of the instant claims.[24] Chang then discloses *thousands* of genes from a variety of plant species and suggests that either up-regulating *or* down-regulating these genes may lead to improved resistance to various plant pathogens.[25] Furthermore, even if Chang did specifically teach the down-regulation of the ParG gene—which it does not—Chang's SEQ ID No. 550 is *not* identical to instant SEQ ID No. 3, as alleged in the Office Action.[26] SEQ ID No. 3 is 1647 nucleotides in length, whereas SEQ ID No. 550 of Chang is 2994 nucleotides in length. Indeed, the two sequences are identical only up through the first 521 nucleotides, after which they begin to diverge.

Applicant also respectfully disagrees with the Office Action's characterization of Panda. The Office Action states that "Panda et al. teach the function of ParG…by isolating *Arabidopsis* mutants disrupted in ParG expression."[27] According to the Office Action, these mutants "were tolerant to high light stress."[28] Applicant respectfully submits that Panda provides *absolutely no teaching* of increased tolerance to high light stress in plants with decreased levels of ParG expression. Panda instead discloses that *Arabidopsis* mutants with decreased ParG expression exhibit a lengthened circadian period that is entirely *independent* of the frequency or intensity of

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[22] *Id.*

[23] *See* Office Action, p. 7.

[24] *See* Chang, pp. 34, 57-58, and 98-108; *see also* Office Action, p. 8.

[25] Office Action, pp. 7-13.

[26] Office Action, p. 7 ("Chang…teach[es]…a ParG (poly(ADP-ribose) glycohydrolase) nucleotide sequence as defined in SEQ ID NO: 550 *which is identical in sequence* to instant SEQ ID NO: 3.") (emphasis added).

[27] Office Action, p. 8.

[28] *Id.*

the light to which the mutant is exposed.[29] Applicant is unaware of any disclosure in Panda whatsoever that links decreased ParG expression to increased tolerance to high light stress.

D. The Office Action Fails To Establish A *Prima Facie* Case Of Obviousness

1. The Combination of Chang, Wesley, and Panda Does Not Teach Each and Every Claim Element.

The combination of Chang, Wesley, and Panda fails to establish a *prima facie* case of obviousness. First, the claims require nucleotide sequences that correspond to the amino acid sequence of SEQ ID NO: 1 or the nucleotide sequence of SEQ ID NO: 3. None of the cited references, alone or in combination, teaches or suggests either of these sequences. As discussed above, Chang's SEQ ID NO: 550 has some homology to, but is *not identical to*, SEQ ID NO: 3. Wesley and Panda also fail to provide any disclosure of SEQ ID NO: 1 or SEQ ID NO: 3. Because this critical element of the claims is not taught by any of the references, a *prima facie* case of obviousness has not been established.

Second, the references also fail to provide any teaching whatsoever regarding the down-regulation of ParG to achieve increased tolerance to high light stress. Chang is directed to manipulating thousands of different genes to achieve increased resistance to pathogenic infections. The reference is devoid of any teachings relevant to increased tolerance to high light stress. Wesley is likewise silent on the issue of increased tolerance to high light stress. Panda, which the Office Action actually relies upon for disclosure of the link between down-regulation of ParG and increased high light tolerance, is also completely silent regarding tolerance to high light stress. As discussed above, Panda merely reports that mutants with decreased ParG activity have a lengthened circadian period. Indeed, Panda devotes an entire section of discussion to the proposition that the effects of the ParG mutation being studied were *completely independent* of the type (i.e. wavelength) or intensity of the light to which the plants were exposed.[30] Applicant is unaware of any mention of increased tolerance to high light stress in Panda.

For the foregoing reasons, Applicant respectfully submits that the cited references fail to support a *prima facie* case of obviousness and requests withdrawal of the rejection.

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[29] *See* Panda, pp. 52-53 ("The mutant exhibited a constitutively lengthened period over a wide range of fluence rates....[T]he mutant still exhibited a longer free-running period length compared to the WT in absence of any light input to the clock...*conclusively establishing that the effect of the tej [ParG] mutation on circadian period length in Arabidopsis is independent of light input to the clock.*") (emphasis added).

[30] Panda, pp. 52-53.

2. There Is No Reason To Combine Chang, Wesley, and Panda.

One of skill in the art would have had no reason to combine the cited references. As discussed above, Chang describes thousands of genes that are differentially regulated in response to pathogenic infections (i.e., *biotic* stress conditions).[31] Chang does not teach that the ParG gene of SEQ ID NO: 3 is in any way involved in increasing the tolerance of *Arabidopsis*, *Brassica*, or tobacco plants to high light stress. Instead, Chang teaches that the up-regulation *or* down-regulation of SEQ ID NO: 550—along with thousands of other genes disclosed by Chang—*may* help improve a plant's ability to survive pathogenic infections. Chang contains no indication as to whether the expression of the gene encoded by SEQ ID NO: 550 should be up-regulated or down-regulated to enhance pathogen resistance in plants, let alone whether such an alteration in gene activity would have any effect on tolerance to high light stress. Because Chang provides no specific teaching suggesting that ParG should be down-regulated, one of skill in the art would not have sought to combine Chang with Wesley, which provides a general disclosure of the method of RNAi for inhibiting gene expression.

One of skill in the art would also have had no motivation to combine Chang and Wesley with Panda. Neither Chang nor Wesley provides any specific teaching of the desirability of down-regulating Chang's SEQ ID NO: 550, let alone the ParG gene of SEQ ID NO: 3. Accordingly, one of skill in the art would have had no reason to consider Panda, which discloses mutant plants with reduced ParG gene activity. However, even if one of skill in the art had been motivated to consider Panda, the reference fails to provide any teaching that the down-regulation of ParG provides either increased tolerance to high light stress or increased resistance to pathogenic infections. Instead, Panda discloses that plants with reduced ParG activity have a lengthened circadian period. The reference is completely silent as to whether this reduced ParG activity confers any tangible benefit (e.g. increased tolerance to either pathogenic or high light stress) on plants. Therefore, one of skill in the art would have had no reason to inhibit ParG expression to obtain plants tolerant to high light stress.

3. Panda Teaches Away From The Claimed Invention

To the extent that Panda is at all relevant to the instant invention, Applicant submits that the reference actually *teaches away* from the down-regulation of ParG to increase tolerance to high light stress. The only reference to "stress" in Panda is found on page 57, which explains that the

[31] *See* Chang, pp. 7-9.

PARP gene (*not* the ParG gene) is generally induced in response to cellular stress.[32] PARP catalyzes the poly(ADP-ribosyl)ation of certain proteins, whereas ParG catalyzes the *opposite* reaction—removing the poly(ADP-ribose) polymers from these tagged proteins. Significantly, it is known that *down-regulation* of PARP can function to enhance stress tolerance in plants.[33] Accordingly, one of skill in the art would not have expected that the down-regulation of ParG, a protein whose activity *opposes the activity of PARP*, would confer tolerance to high light stress in plants. Rather, one of skill in the art would have been motivated to *up-regulate* ParG activity to counter PARP activity in order to achieve increased stress tolerance. Thus, Panda's reference to PARP's role in the plant stress response actually teaches away from the instant invention.

In view of the foregoing, Applicant respectfully requests withdrawal of the rejection.

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[32] Panda, p. 57 ("PARP is acutely induced under genotoxic stress, and its activity plays a critical role in modulating cellular response to stress.").

[33] *See* Specification, ¶ [8] (describing method of WO 00/04173); *see generally* De Block (Exhibit A).

CONCLUSION

Applicant respectfully submits that the pending claims are in condition for allowance, and such disposition is earnestly solicited. Should the Examiner believe that any issues remain after consideration of this Response, the Examiner is invited to contact the Applicant's undersigned representative to discuss and resolve such issues.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Date: August 26, 2009

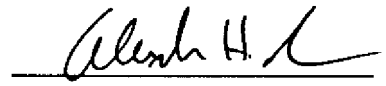
By: 

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EXHIBIT A

De Block M, Verduyn C, De Brouwer D, Cornelissen M, "Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance", Plant J. 41:95-106, 2005.

The Plant Journal (2005) 41, 95–106 doi: 10.1111/j.1365-313X.2004.02277.x

Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance

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Summary

Plants contain two genes that code for poly(ADP-ribose) polymerase (PARP): *parp1* and *parp2*. Both PARPs are activated by DNA damage caused by, example reactive oxygen species. Upon activation polymers of ADP-ribose are synthesized on a range of nuclear enzymes using NAD^+ as substrate. Here, we show that in plants stresses such as drought, high light and heat activate PARP causing NAD^+ breakdown and ATP consumption. When the PARP activity is reduced by means of chemical inhibitors or by gene silencing, cell death is inhibited and plants become tolerant to a broad range of abiotic stresses like high light, drought and heat. Plant lines with low poly(ADP-ribosyl)ation activity maintain under stress conditions their energy homeostasis by reducing NAD^+ breakdown and consequently energy consumption. The higher energy-use efficiency avoids the need for a too intense mitochondrial respiration and consequently reduces the formation of reactive oxygen species. From these results it can be concluded that breeding or engineering for a high energy-use efficiency under stress conditions is a valuable, but until today nearly unexploited, approach to enhance overall stress tolerance of crops.

Keywords: cell death, energy metabolism, mitochondrial respiration, NAD^+, poly(ADP-ribose) polymerase, stress tolerance.

Introduction

Over the past decades breeders have successfully improved the maximum attainable yield of the main field crops. These yield improvements are partly or, as in corn, primarily attributable to the enhancement of the overall stress tolerance (Duvick, 1997; Tollenaar and Lee, 2002; Tollenaar and Wu, 1999). Despite this steady progress, today still only 30–70% of the attainable yield is actually realized. This is due to unfavourable growth conditions such as nutrient shortage, cold nights, drought at the young seedling stage, high temperatures at flowering time, etc. This difference between the maximum attainable yield and the average farmer yield is referred to as the 'yield gap' (Dobermann *et al.*, 2003; Murrell and Childs, 2000). To narrow the yield gap the growth optimum of the crop for many growth parameters and their combinations should be broadened. Both breeding and biotechnology are nowadays exploited to reduce the yield gap. In breeding, varieties are being selected by their performance in different geographical locations over several years, whereas in biotechnology the recent insights into the mode of action of numerous genes are being exploited to engineer stress tolerance. By enhancing the tolerance to multiple stresses, plants become less sensitive to adverse environmental changes and this broadens indirectly the growth optimum. Because most types of stresses induce the production of reactive oxygen species, the overproduction of detoxification enzymes such as superoxide dismutases was one of the first approaches that have been evaluated to enhance the overall stress tolerance in plants (Alscher *et al.*, 2002). In addition, the overproduction of compatible solutes such as trehalose (Garg *et al.*, 2002; Jang *et al.*, 2003) and glycine betaine (Chen and Murata, 2002) result in stress-protective effects, which is due to the ability to scavenge reactive oxygen species besides their chaperon-like activity. Most of the current stress research is focused on understanding the stress signalling pathways. Many of the transcription factors (Chinnusamy *et al.*, 2003; Dubouzet *et al.*, 2003; Gilmour *et al.*, 2000; Kasuga *et al.*, 1999; Knight and Knight, 2001; Xiong *et al.*, 2002; Zhu, 2001) and more recently components of the mitogen-activated protein kinase signal transduction pathway (Meskiene and Hirt, 2000; Moon *et al.*,

2003a; Shou *et al.*, 2004) have been identified. Numerous reports show that stress tolerance may be improved by overexpressing these components. For comprehensive overviews about the current state of biotechnology in stress research we refer to Datta (2002) and Wang *et al.* (2003). A very different but promising approach to promote broad stress tolerance is by blocking stress-induced cell death. By overexpression of animal cell death suppressors such as Bax-inhibitors, Bcl2, Bcl-X_L, DAD-1, and IAP plants with improved tolerance to both necrotrophic pathogens and abiotic stresses such as UV, cold, salt and reactive oxygen species have been obtained (Awada *et al.*, 2003; Dickman *et al.*, 2001; Lincoln *et al.*, 2002; Moon *et al.*, 2003b; del Pozo and Lam, 2003; Qiao *et al.*, 2002). The same is true when the plant-derived anti-apoptotic genes such as BI-1, nucleoside diphosphate kinase 2 and DAD-1 were overexpressed in plants (Bolduc *et al.*, 2003; Hoeberichts and Woltering, 2003; Kawai-Yamada *et al.*, 2001; Matsumura *et al.*, 2003).

In this paper we describe a very different strategy to broaden stress tolerance in plants, by maintaining energy homeostasis under stress conditions. Today, there is no clear picture about the influence of stresses on energy metabolism, but in general stresses cause high energy consumption and enhance the respiration with a linked production of reactive oxygen species (Rizhsky *et al.*, 2002; Tiwari *et al.*, 2002). When the stresses are extreme or persistent an energy threshold is reached at which the damage caused by the stress can no longer be repaired, and the cell, tissue or ultimately the whole plant will die. This would predict that when stress-induced energy consumption could be reduced by enhancing the energy-use efficiency, the plant could overcome peak stresses or have the opportunity to acclimate to moderate but persistent stresses. The major stress-induced energy-consuming processes in plants are not well known, but in animals the stress-induced activation of poly(ADP-ribose) polymerase is the main cause of energy depletion. The enzymes PARP1 [Poly(ADP-Ribose) Polymerase-1], and to a lesser extent PARP2 [Poly(ADP-Ribose) Polymerase-2], are mainly responsible for the stress-induced poly(ADP-ribosyl)ation activity in animals. Both PARPs are activated by DNA damage caused by, example radicals (Virág and Szabó, 2002). Upon activation polymers of ADP-ribose are synthesized on a range of nuclear enzymes using NAD^+ as substrate (Bakondi *et al.*, 2002). Overactivation of PARP, as occurs in ischaemia, inflammation and neural diseases as Alzheimer's, causes a rapid breakdown of the NAD^+ pool (Du *et al.*, 2003; Klaidman *et al.*, 2003; Nakamura *et al.*, 2003; Virág and Szabó, 2002). As a consequence resynthesis of NAD^+ is stimulated whereby three (NAD^+-salvage pathway) to five (*de novo* synthesis) molecules of ATP are used for each molecule of NAD^+. In this way the cellular ATP is depleted which leads to necrotic cell death (Filipovic *et al.*, 1999; Ha and Snyder, 1999; Virág and Szabó, 2002). PARP1 is a 116-kDa nuclear enzyme composed of three functional domains: an N-terminal DNA-binding domain containing two zinc-finger motifs, a central automodification domain and a C-terminal catalytic domain, which is the most conserved region between PARP1 and PARP2. The catalytic domain contains a very conserved block of 50 amino acids, referred to as the PARP signature. PARP2 is a 62-kDa enzyme and is also located in the nucleus. It is composed of an N-terminal DNA-binding domain, however, without zinc-fingers, and a C-terminal catalytic domain containing the PARP signature (Virág and Szabó, 2002).

In plants, PARP1 and PARP2 homologues are found with a very similar structure to their animal counterparts (Babiychuk *et al.*, 1998). Both PARPs are localized in the nucleus and are activated by DNA strand breaks (Babiychuk *et al.*, 1998; Chen *et al.*, 2003; Doucet-Chabeaud *et al.*, 2001; Puchta *et al.*, 1995). Experiments in Arabidopsis have shown that DNA strand breaks, caused by ionizing radiation or oxidative stress, induce rapid and massive accumulation of both *parp1* and *parp2* transcripts in all plant tissues. The *parp2* gene is also induced by different kinds of environmental stresses such as drought and heavy metals (Doucet-Chabeaud *et al.*, 2001). Amor *et al.* (1998) showed that in cultured soybean cells expressing antisense *parp2* mRNA, H_2O_2-induced cell is inhibited.

In this paper we provide evidence for a role of PARP in energy homeostasis and stress tolerance. Our data show that in plants strong stresses induce poly(ADP-ribosyl)ation-activity causing NAD^+ breakdown and an enhanced mitochondrial respiration. By reducing stress-induced poly(ADP-ribosyl)ation-activity NAD^+ breakdown is inhibited preventing high energy consumption. Under these conditions plants preserve their energy homeostasis and this without an overactivation of the mitochondrial respiration, avoiding the production of reactive oxygen species. In this way, plants with a lowered poly(ADP-ribosyl)ation activity appear tolerant to multiple stresses.

Results

PARP inhibitors enhance the tolerance of Brassica napus *hypocotyl explants to oxidative stress*

In animals poly(ADP-ribosyl)ation has a major role in DNA repair, gene expression, cell cycle regulation, recombination, and cell death. Except for yeast, PARP enzymes have been found in all studied eukaryotes.

As a first approach to decipher the role of PARP in plants, the effect of a small panel of chemical inhibitors on the growth of *Brassica napus* callus was studied. Three PARP-inhibitors were used: 3-methoxybenzamide (3MB), nicotinamide (NA), and isonicotinamide (INA). Animal PARP1 is inhibited at 1 mM concentrations with 92% by 3MB, 72% by NA and 51% by INA (Banasik *et al.*, 1992). Figure 1 shows the

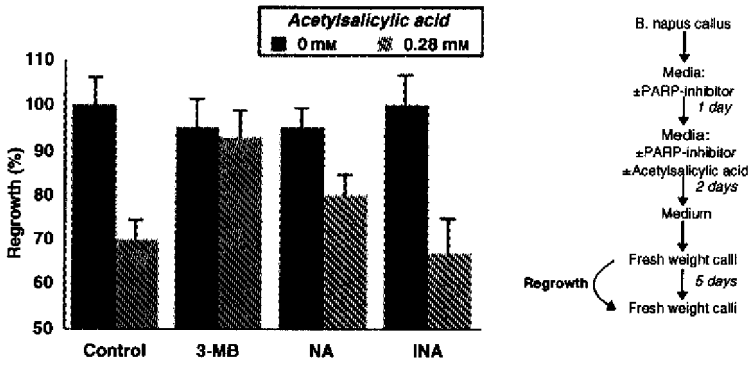

Figure 1. PARP inhibitors protect *Brassica napus* callus against oxidative stress. *Brassica napus* callus was incubated for 2 days on medium containing 0.28 mM acetylsalicylic acid. The regrowth of the callus was scored after another 5 days. The PARP inhibitors 3-methoxybenzamide (3-MB), nicotinamide (NA) and isonicotinamide (INA) were used at 1 mM concentrations. The regrowth of the non-treated callus (no PARP inhibitors, no acetylsalicylic acid) was set at 100%. Error bars indicate the standard error ($n = 3$).

layout and results of an experiment in which the impact of 3MB, NA and INA on the regrowth of *B. napus* callus was measured after 2 days of oxidative stress induced by acetylsalicylic acid (De Block and De Brouwer, 2002). When 3MB was used callus regrowth was hardly inhibited by oxidative stress. The weaker inhibitor NA had some protective effect, while the weakest inhibitor INA did not protect the tissue against acetylsalicylic acid. These observations indicate that PARP inhibitors protect explants in tissue culture against oxidative stress and disclose a relationship between PARP activity and stress sensitivity.

Overexpression of dsRNA-parp constructs enhances the overall stress tolerance of plants

As PARP inhibitors are not completely specific and potentially also inhibit other enzymes (Banasik *et al.*, 1992; Cosi, 2002; Southan and Szabó, 2003) a genetic approach was used to understand the role and function of PARP in stress tolerance. Arabidopsis and oilseed rape were transformed with dsRNA constructs containing the 5′-end of the Arabidopsis *AtParp1* or *AtParp2* genes in the stem structure (Waterhouse *et al.*, 1998, 2001). These constructs are indicated as *hpAtParp1* and *hpAtParp2* (hp stands for hairpin). Segregating populations for the hpRNA constructs obtained by selving or backcrossing of the heterozygous lines, were tested for tolerance to various stresses like drought, heat, and high light. The phenotype and growth of the transgenic plants were compared with their azygous counterparts. Of 50 lines per construct and plant species, 10% of the hpAtParp1 and 20% of the hpAtParp2 lines were very tolerant to the applied stresses, while another 20% of the hpAtParp1 and 30% of the hpAtParp2 lines was partly stress tolerant. Figure 2(a,b) shows representative phenotypes that were observed in stress experiments. The graph in Figure 2(d) depicts at the end of a drought experiment the fresh weight of the transgenic and azygous plants of 1:1 segregating Arabidopsis populations for the *hpAtParp* transgenes. The weight of the azygous plants was similar to these of the non-transgenic control. The plants transgenic for the *hpAtParp1* or *hpAtParp2* construct had on average 70% higher fresh weight than their azygous counterparts. Lines that were tolerant to drought were also tolerant to methyl viologen (Figure 3c). The frequencies with which stress tolerant lines were obtained indicate that downregulation of *parp2* is more effective than downregulation of *parp1*.

The impact of both *parp1* and *parp2* on stress tolerance was studied in an experiment with a similar set-up as outlined in Figure 2(b). By crossing a transgenic Arabidopsis line was made containing *hpAtParp1* and *hpAtParp2* loci that each provided stress tolerance in the parental lines (Figure 2b–d). The stress tolerance of the (hpAtParp1 + hpAtParp2) line was compared with the parental lines. The graph in Figure 2(e) shows that overexpression of both the *hpAtParp1* and *hpAtParp2* genes results in a higher stress tolerance than overexpression of only one of the *hpAtParp* genes.

This finding was further supported by studies with Arabidopsis lines carrying an hpRNA construct targeting the sequence of the *AtParp2* catalytic domain around the PARP signature (Amé *et al.*, 1999; Babiychuk *et al.*, 1998). This region has an overall sequence similarity of 86% between *AtParp1* and *AtParp2*, with a block of 24 bp with 100% sequence similarity at the PARP-signature. This construct, indicated as *hpAtParp2(signature)*, would allow silencing of both *AtParp1* and *AtParp2* (Holzberg *et al.*, 2002). Up to 40% of the hpAtParp2(signature) lines were tolerant to high light stress, while this was only 10% of the hpAtParp1 and 20% of the hpAtParp2 lines.

The stress-tolerant hpParp-lines have under stress conditions a reduced poly(ADP-ribosyl)ation activity

The activity of poly(ADP-ribose) polymerase results in the poly(ADP-ribosyl)ation (PAR) of nuclear proteins (Affar *et al.*, 1998; Bakondi *et al.*, 2002). This allowed us to test to what extent the observed stress tolerance correlates with the PAR activity. During high light stress an increase in poly (ADP-ribosyl)ated proteins was detected in the nuclei of wild-type Arabidopsis plants with a peak 4–6 h after start (Figure 3a). The hpAtParp1, hpAtParp2 and hpAtPARP2

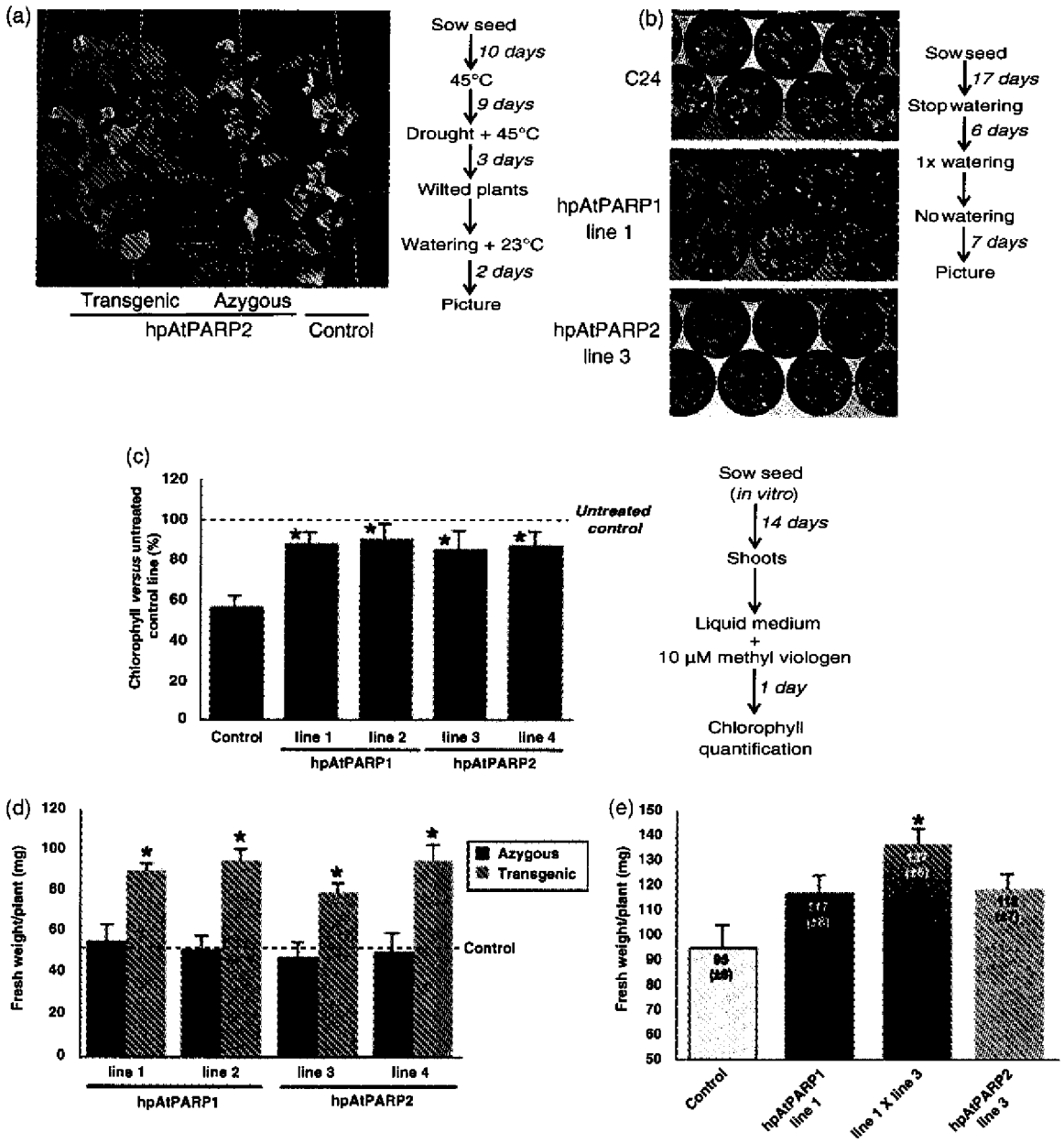

Figure 2. Overexpression of the *hpAtParp* constructs enhances the stress tolerance of plants.
(a) Phenotypes of a control and a 3 transgenic:1 azygous segregating *Brassica napus* hpAtParp2 line at the end of a stress experiment where heat and drought had been combined.
(b) Phenotypes of *Arabidopsis thaliana* cv. C24 lines at the end of a drought stress experiment.
(c) Chlorophyll content of *A. thaliana* cv. C24 untransformed control and homozygous hpAtParp lines treated with 10 µM methyl viologen for 1 day.
(d) Fresh weight of *Arabidopsis thaliana* cv. C24 plants from a 1 transgenic:1 azygous segregating hpAtParp1 and hpAtParp2 lines at the end of a drought stress experiment as outlined in Figure 2(b). The segregating lines for the *hpAtParp* constructs were obtained by backcrossing hemizygous hpAtParp plants with wild-type plants.
(e) The fresh weight of *Arabidoposis thaliana* cv. C24 plants at the end of a drought experiment as outlined in Figure 2(b). The line containing both the *hpAtParp1* and *hpAtParp2* constructs was obtained by crossing the homozygous hpAtParp1 line 1 with the homozygous hpAtParp2 line 3 (b–d). The numbers in the bars are the mean and the standard error. The hpAtParp lines 1 to 4 are the same in (b–e). The error bars indicate the standard error ($n = 3$). *Significant difference at $P = 0.01$.

(signature) lines that were scored as stress tolerant (Figures 2b–d and 5c–e) had low PAR activities, while the control and stress sensitive lines had high PAR activities, especially when stressed by high light (Figure 3b,c). These data strongly argue that the enhanced stress tolerance of the hpAtParp lines correlates with a low PAR activity at stress conditions.

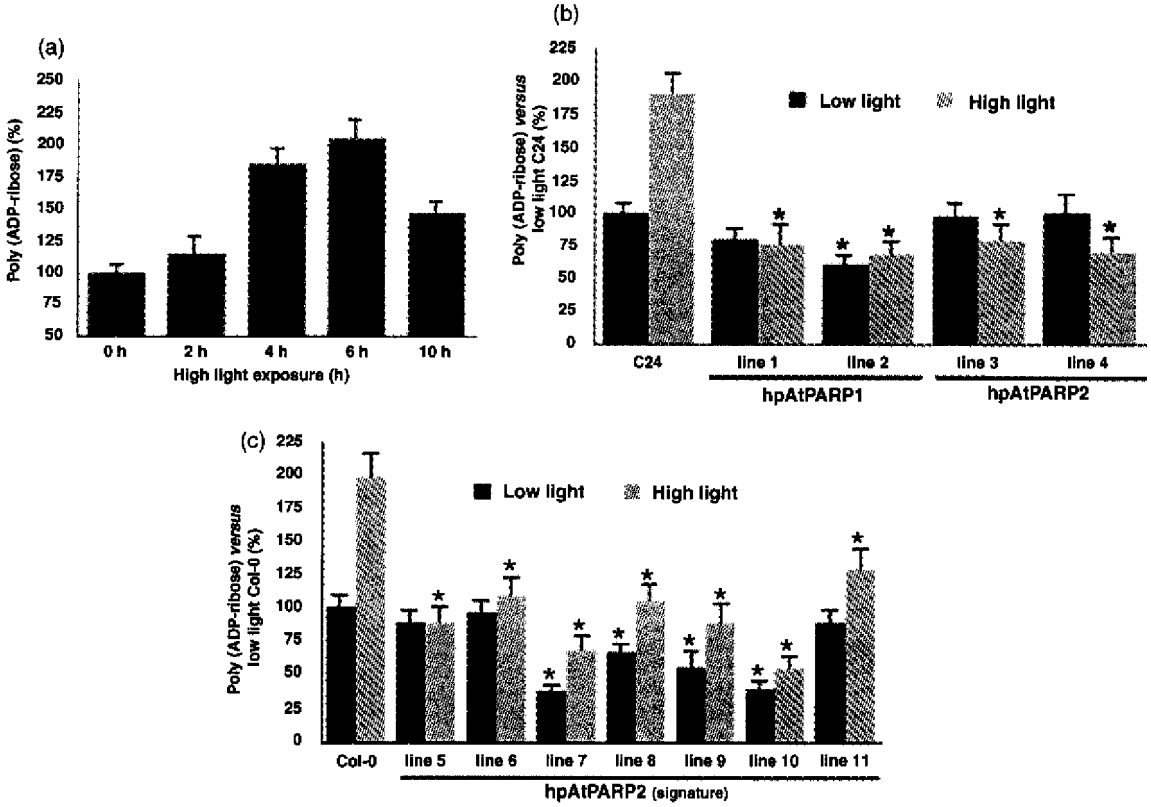

Figure 3. The hpAtParp lines have a reduced poly(ADP-ribosyl)ation activity (PAR).
(a) *Arabidopsis thaliana* cv. Col-0 was stressed by high light. Samples were taken at various time points and the PAR activity was determined.
(b) PAR activities of *Arabidopsis thaliana* cv. C24 wild-type and stress-tolerant hpAtParp1 or hpAtParp2 lines (Figure 2b–d) stressed by high light.
(c) PAR activities of *Arabidopsis thaliana* cv. Col-0 lines stressed by high light. The lines are transgenic for the *hpAtParp2(signature)* construct and have different tolerance levels to high light stress (Figure 5c–e): lines 6 and 11 are sensitive; lines 7 and 10 are tolerant; lines 8 and 9 have an intermediate tolerance. Low light (30 μEinstein m^{-2} sec^{-1}); high light (220 μEinstein m^{-2} sec^{-1}). The error bars indicate the standard error ($n = 3$). *Low and high light mean values are statistically different from the control at $P = 0.01$.

Inhibition of PARP reduces stress-induced cell death

In animals activation of PARP1 results in NAD^+ breakdown, ATP overconsumption and finally in energy depletion causing necrotic cell death, whereas inhibition of PARP activity leads to maintenance of the NAD^+ and ATP levels under stress conditions and a reduced cell death (Filipovic *et al.*, 1999; Ha and Snyder, 1999; Virág and Szabó, 2002). We also tested whether in plants inhibition of PARP would reduce stress-induced cell death. Both programmed and necrotic cell death are characterized by the degradation of nuclear DNA and can be visualized with the TUNEL assay that labels the DNA breaks (Gavrieli *et al.*, 1992). Figure 4 shows the result of an *in situ* TUNEL assay carried out on the top ends of *B. napus* hypocotyl explants incubated for 1 day in medium containing acetylsalicylic acid. Numerous nuclei were labelled in the cortex tissue of explants derived from control or azygous plants, while only few nuclei were labelled in explants derived from stress-tolerant transgenic hpAtParp plants. Similar results were obtained by treating explants derived from non-transgenic plants with the PARP-inhibitor 3-methoxybenzamide 1 day before and during the 1-day incubation in acetylsalicylic acid-containing medium. These data indicate that in plants inhibition of PARP protects the cells against stress-induced cell death (necrotic and/or programmed).

Inhibition of PARP prevents energy overconsumption in stress conditions

The above similarities between the role of PARP in animals and plants prompted us to investigate further parallels. We therefore tested whether the hpAtParp lines showed altered energy housekeeping. To this end, *B. napus* hypocotyl explants were cultured on medium containing 0.06 M glucose as carbon source. Glucose is a suboptimal carbon source for *in vitro* cultured *B. napus* hypocotyl explants, and

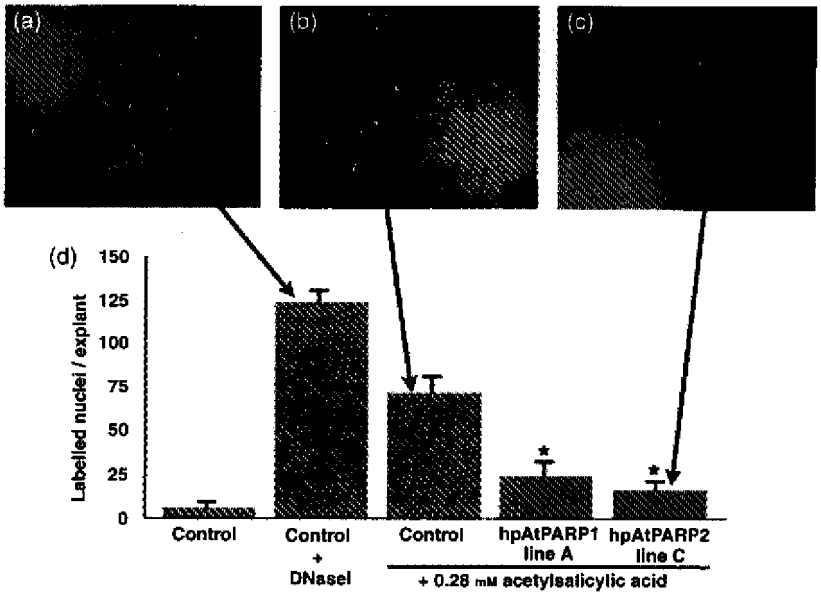

Figure 4. Stress-induced cell death is inhibited in the hpAtParp lines. TUNEL assay carried out on the top ends of 5-day-cultured *Brassica napus* hypocotyl explants.
(a) Control explants treated for 1 h at 37°C with 40 U ml^{-1} DNaseI. Most nuclei are labelled.
(b) Explants from a non-transgenic control incubated for 1 day in medium containing 0.28 mM acetylsalicylic acid.
(c) Explants from an hpAtParp2 line incubated for 1 day in medium containing 0.28 mM acetylsalicylic acid. The red arrows in (a–c) indicate examples of labelled nuclei.
(d) Quantification of the number of labelled nuclei/explant. The nuclei that were labelled in the cortex tissue of the explants were counted. In each experiment 50 explants/line and treatment were scored. The lines A and C correspond with lines A and C of Table 1. The error bars indicate the standard error ($n = 3$). *Mean values are statistically different from the acetylsalicylic acid treated control at $P = 0.01$.

concentrations higher than 0.1 M glucose have to be used to allow a good callus induction and growth. Figure 5(a) shows 3-week-old hypocotyl explants that were cultured on medium containing 0.06 M glucose. The explants of the control line are necrotic and have a poor callus formation, while the explants of the hpAtParp2 lines are still green and form vigorous callus. This is further illustrated in the graph of Figure 5(b) that shows the phenotype and weight of the hypocotyl explants of stress-sensitive and -tolerant hpAtParp lines that were cultured for 3 weeks on 0.06 M glucose. The stress-tolerant hpAtParp lines produced more callus and the hypocotyl explants did not become necrotic. However, when the seeds were germinated on medium containing 2–6% glucose no difference in seedling growth could be measured between the hpAtParp lines and the non-transgenic control line. This indicates that the differential callus formation and survival of the explants on glucose medium between the stress-tolerant hpAtParp lines and the control line is not due to an altered sugar sensing. Similar seedling-growth experiments with the Arabidopsis hpAtParp lines confirmed that downregulation of the *parp* genes does not alter sugar sensing. In summary, the above experiments suggest that reducing the PAR activity results in higher energy efficiency under stress conditions.

To test whether energy homeostasis is the basis of the stress tolerance of the hpAtParp lines, the total NAD^++NADH and ATP content were quantified under control and stress conditions. A non-transgenic control and four hpAtParp2(signature) Arabidopsis lines were stressed by high light. The four hpAtParp2(signature) lines had different levels of stress tolerance towards high light going from a tolerance comparable to the non-transgenic control line to a high stress tolerance (Figure 5e). The graph in Figure 5(c) shows that when the lines were more stress tolerant the total NAD^++NADH content was less affected by stress. This is also reflected in the ATP content that in the stress tolerant lines even increased by stress. This implies that energy homeostasis is at the basis of the observed stress tolerance.

Preventing energy overconsumption allows a normal mitochondrial respiration

Most stresses interfere with a normal mitochondrial function. This interference results in a high radical production that causes cell damage. When *B. napus* hypocotyl explants were incubated in medium containing 0.28 mM acetylsalicylic acid only a minor increase in superoxide production was measured in the explants derived from the hpAtParp lines: about 3–8% increase in the hpAtParp lines versus about 167% in the controls (Table 1). This pointed to an efficient mitochondrial electron transport in the hpAtParp lines that was further confirmed by the high ratio of moles ATP present in the explants to the respiration rate.

The high energy status, the efficient cellular respiration and the low radical production indicate that there is under stress lower energy consumption and by this a lower respiration rate in the hpAtParp lines when compared with the controls. This prediction was tested by measuring the capacity of the lines to reduce 2,3,5-triphenyltetrazolium-chloride (TTC). TTC is reduced by the mitochondrial electron transport system to an insoluble formazan that can be extracted from the cells and tissues by ethanol and subsequently quantified spectrophotometrically (De Block and De Brouwer, 2002). Figure 5(d) shows the results of a TTC assay on high light-stressed Arabidopsis lines. The stress-tolerant

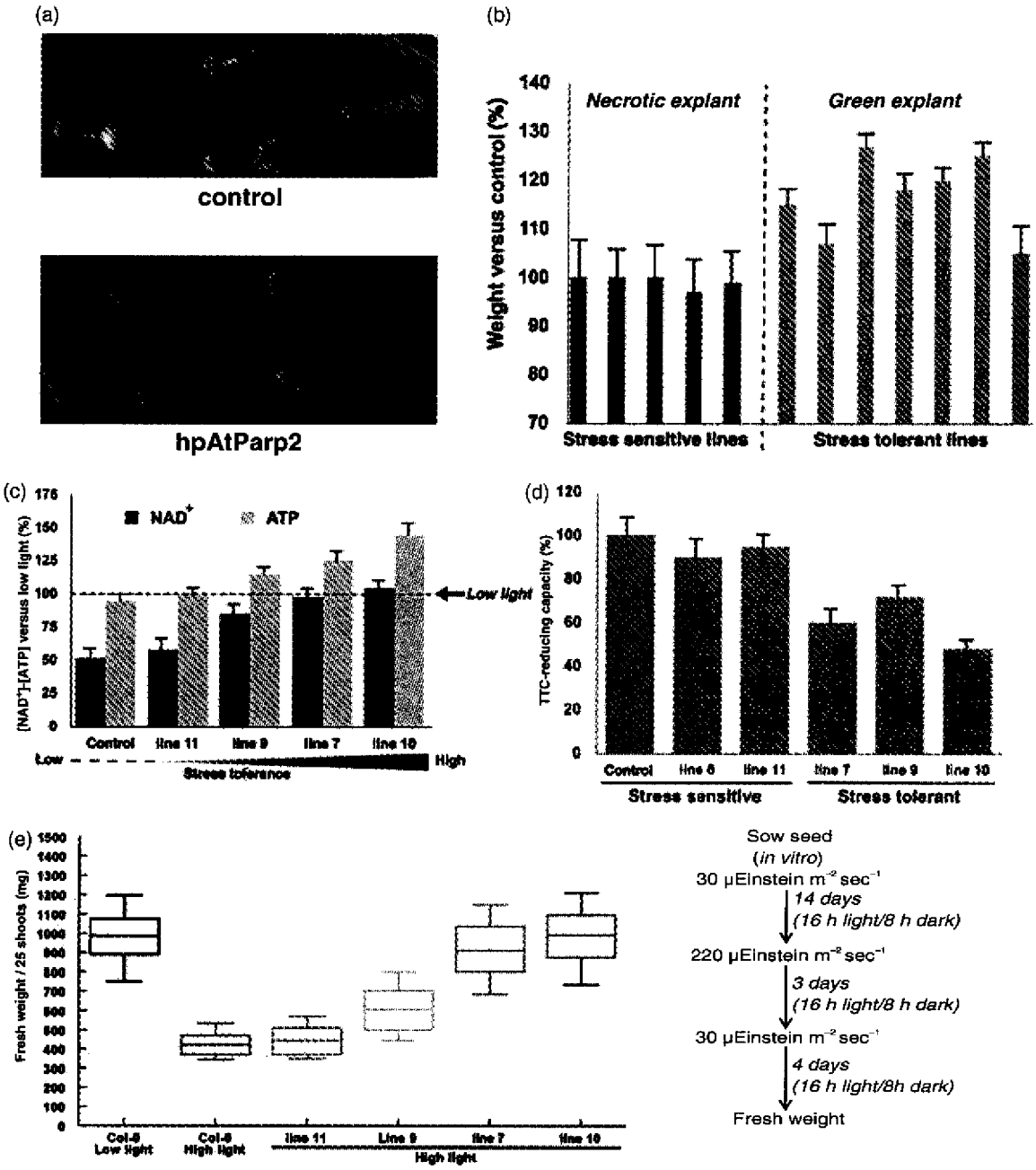

Figure 5. The hpAtParp lines maintain their energy homeostasis under stress conditions.
(a) Phenotypes of *Brassica napus* hypocotyl explants cultured for 2 weeks on callus inducing medium containing 0.06 M glucose as carbon source. The red arrow indicates a necrotic explant.
(b) Fresh weight of *B. napus* hypocotyl explants cultured for 2 weeks on callus inducing medium containing 0.06 M glucose as carbon source. The callus weight of the non-transgenic control line was set at 100%. The error bars indicate the standard error ($n = 3$).
(c) NAD^++NADH and ATP contents of control and hpAtParp2(signature) *Arabidopsis thaliana* cv. Col-0 lines that have been stressed for 24 h by high light. The NAD^++NADH and ATP contents of the non-stressed control line were set at 100% and are indicated by the dashed line 'low light' (30 µEinstein m^{-2} sec^{-1}). The lines are sorted from low to high stress tolerance as scored in high light experiments (e).
(d) Quantification of mitochondrial electron transport by measuring TTC reduction. TTC-reducing capacity of control and hpAtParp2(signature) *A. thaliana* cv. Col-0 lines. The plants were stressed for 24 h by high light. Error bars indicate standard error ($n = 3$).
(e) Tolerance of hpAtParp2(signature) lines to high light stress. High light stress was applied as described in the scheme. Fresh weight was determined per 25 shoots. For each experiment 100 shoots per line and condition were used. The lines in the boxes represent the mean, the lower and upper edges of the box the 25th and 75th percentiles, and the whiskers the 10th and 90th percentiles.

Table 1 Influence of acetylsalicylic acid on energy and radical production of 5 days cultured *B. napus* hypocotyl explants

Line	Stress	Moles ATP[a] / mg consumed O_2^b (SE)[c]	% superoxide production versus control (SE)[c]
Control	None	4.19×10^{-6} ($\pm 0.25 \times 10^{-6}$)	–
	ASA[d]	2.85×10^{-6} ($\pm 0.25 \times 10^{-6}$)	+167 (±13)
hpAtParp1 (line A)	None	3.99×10^{-6} ($\pm 0.21 \times 10^{-6}$)	–
	ASA[d]	4.04×10^{-6} ($\pm 0.28 \times 10^{-6}$)	+8 (±2)
hpAtParp1 (line B)	None	4.10×10^{-6} ($\pm 0.19 \times 10^{-6}$)	–
	ASA[d]	4.25×10^{-6} ($\pm 0.27 \times 10^{-6}$)	+4 (±2)
hpAtParp2 (line C)	None	4.07×10^{-6} ($\pm 0.24 \times 10^{-6}$)	–
	ASA[d]	4.22×10^{-6} ($\pm 0.33 \times 10^{-6}$)	+4 (±2)
hpAtParp2 (line D)	None	4.15×10^{-6} ($\pm 0.22 \times 10^{-6}$)	–
	ASA[d]	4.28×10^{-6} ($\pm 0.25 \times 10^{-6}$)	+3 (±2)

[a] g^{-1} fresh weight.
[b] h^{-1} g^{-1} fresh weight.
[c] SE ($n = 3$).
[d] Explants were incubated for 1 day in medium containing 0.28 mM acetylsalicylic acid.

lines have a lower TTC-reducing capacity/respiration than the stress-sensitive lines.

Discussion

In this paper we described the utility of reducing poly(ADP-ribosyl)ation activity to engineer broad stress tolerance in plants. Our data show that: First, plants with reduced PAR-activity are tolerant to a broad range of stresses. Secondly, stress tolerance is obtained by maintaining energy homeostasis. Thirdly, energy homeostasis is maintained by reducing stress-induced energy consumption by preventing NAD^+ breakdown. This results in higher energy-use efficiency. Fourthly, reducing energy consumption avoids a too intense mitochondrial respiration and consequently prevents the formation of reactive oxygen species.

These data point to the importance of the cellular energy metabolism in relation to stress in plants. In animals energy depletion due to mitochondrial dysfunction and/or by energy consuming processes may lead to both programmed and necrotic cell death (Ricci *et al.*, 2003). When the ATP content of a cell drops below a certain threshold mitochondrial permeability transition pores are formed. This causes the release of cell death initiators and the breakdown of ATP by the mitochondrial ATPase. Free radicals and reactive oxygen species may damage the mitochondria directly by attacking the mitochondrial enzymes and the electron transport chain and by opening the mitochondrial permeability transition pores. In this way the energy production process is disrupted. The combination of high energy consumption with a poor ATP production will finally result in cell death. The theory that PARP upon activation is one of the major energy consumers and that overactivation of PARP results in cell death by energy depletion has been confirmed by numerous studies. In most, but not all studies, it has been proved that pharmacological inhibition or genetic inactivation of PARP prevents energy depletion and necrotic cell death (Filipovic *et al.*, 1999; Ha and Snyder, 1999; Virág and Szabó, 2002). It has also been observed that even a moderate PARP activation may decrease the cellular NAD^+ content sufficient to compromise the cellular energy status. This does not cause cell death but a dysfunction of the cell. In these cases pharmacological inhibition of PARP improves cellular energetics and restores cell function (Virág and Szabó, 2002).

Although the knowledge about cellular energetics in regard to stress is less elaborated in plants, it is known that oxidative stress increases cellular respiration, interferes with mitochondrial energy production and causes ATP depletion (Tiwari *et al.*, 2002). In this paper we showed that in plants PARP is a major energy consumer under stress conditions, and that both pharmacological and genetic inhibition of PARP reduces stress-induced energy consumption, protects plants against stress or enables plants to recover from stress injury, and prevents cell death. As in animals the PARP-induced cell death is probably necrotic. Although the nuclei of stressed explants could be labelled by means of the TUNEL assay (Figure 4), that allows to detect both necrotic and programmed cell death, the typical hallmarks for programmed cell death as DNA laddering and cell shrinkage could not be observed. While in animals PARP1 is more abundant than PARP2 and is responsible for 90% of the total PARP activity, this is not the case in plants. Doucet-Chabeaud *et al.* (2001) showed that in Arabidopsis both *parp1* and *parp2* are equally induced by DNA breaks, while stresses as dehydration and heavy metals mainly induce *parp2*. This is in accordance with our results. First, stress tolerance is obtained by reducing PARP1 or PARP2 activity. Secondly, in general, downregulation of PARP2 is more effective for obtaining stress tolerance.

In our study we mainly elucidated the 'pathological' site of PARP. The biological function of PARP in plants is not yet clear, but experimental evidence indicates that PARP is involved in DNA repair also in plants (Puchta *et al.*, 1995).

The presence of *parp* genes in all eukaryotes except yeast and the conserved structure of the enzymes points to their importance for a normal functioning of the organism. Mice knockout lines for *parp1* or *parp2* are defective in DNA excision repair and are hypersensitive to alkylating agents (Schreiber *et al.*, 2002; Trucco *et al.*, 1998) while double knockouts for both *parp1* and *parp2* are not viable (Ménissier de Murcia *et al.*, 2003). To date, we did not find in the hpAtParp-lines any negative effect of downregulation, nor in growth or seed set. We tested the most stress-tolerant Arabidopsis lines for sensitivity to the alkylating agent EMS, but no higher mutation frequency versus the non-transgenic control line was found (data not shown). However, as can be seen in Figure 3, in the stress-tolerant lines the poly(ADP-ribosyl)ation activity is not completely downregulated. Probably, the remnant activity still allows a normal DNA repair as observed in animals using pharmacological inhibitors (Virág and Szabó, 2002).

In conclusion, reducing poly(ADP-ribosyl)ation activity in plants confers tolerance to a broad range of abiotic stresses, by maintaining energy homeostasis. These findings indicate that breeding or engineering for higher energy-use efficiency could be a valuable approach to enhance overall stress tolerance in crops.

Experimental procedures

Plasmid constructs

The *hpAtParp1* construct consists from 5′ to 3′: the cauliflower 35S promoter (Odell *et al.*, 1985) – DNA fragment containing nucleotides 429–1469 of the *AtParp1* cDNA clone – DNA fragment containing nucleotides 428–955 of the *AtParp1* cDNA clone in inverted orientation – 3′ end polyadenylation signal of CaMV35S (Sanfacon *et al.*, 1991). *AtParp1* cDNA clone: GenBank Accession Z48243.

The *hpAtParp2* construct consists from 5′ to 3′: the cauliflower 35S promoter – DNA fragment containing nucleotides 190–1348 of the *AtParp2* cDNA clone – DNA fragment containing nucleotides 189–781 of the *AtParp2* cDNA clone in inverted orientation – 3′ end polyadenylation signal of CaMV35S. *AtParp2* cDNA clone: GenBank Accession AJ131705.

The *hpAtParp2(signature)* construct was made using the pHANNIBAL vector (Helliwell and Waterhouse, 2003). The DNA fragments containing the nucleotides 1572–1730 and 1571–1729 of the *AtParp2* cDNA clone, were cloned in direct inverted orientation.

The *AtParp1* and *AtParp2* cDNA clones were provided by S. Kushnir (Flemish Interuniversity Institute for Biotechnology, Gent, Belgium).

All plasmids constructs contained the *bar* gene (De Block *et al.*, 1987) under the control of the promoter from the Arabidopsis actin-2 gene (Kandasamy *et al.*, 2002) as selectable marker gene.

In vitro *culture of Brassica napus*

The *in vitro* culture of hypocotyl explants was mainly carried out as described (De Block *et al.*, 1989).

In each experiment 150 explants derived from 30 to 40 seedlings were used per line and per condition.

Stress assays

Seeds were sterilized with bleach containing 6% active chlorine and subsequently pre-germinated for 1 day in sterile tap water. Pre-germination reduces the impact of seed vigour on early seedling growth.

The transgenic and azygous plants in segregating populations for the *p35S:hpAtPARP-pact:bar* construct were identified by testing the plants for the presence of the enzyme phosphinothricin acetyl transferase using the Trait LL Leaf/Seed Test Kit of Strategic Diagnostics Inc. (Newark, NJ, USA).

Arabidoposis – high light stress. Plants were grown *in vitro* at 30 μEinstein m^{-2} sec^{-1} (low light) for 2 weeks (16 h light and 8 h dark). The high light stress was applied by transferring the plants to 200–250 μEinstein m^{-2} sec^{-1}.

For quantification of NAD^+, ATP, respiration and radical production, about 60 plants (1–2 g fresh weight) were used per line and condition.

When the tolerance to high light stress on plant growth was studied, 100 plants per line were treated for 3 days (16 h light and 8 h dark) at 200–250 μEinstein m^{-2} sec^{-1}. After the high light treatment the plants were grown for another 4 days. The fresh weight was determined per 25 plants.

Arabidoposis – drought stress. Seedlings were grown for 7–8 days *in vitro* (to obtain homogenous populations) after which they were transferred to flats with 51 pots containing sandy soil. Eight to 9 days after transfer water was withheld for 6 days after which 20 ml water/plant was added once. Results were scored when the azygous and control plants turned yellow, this took another 7–10 days.

Arabidoposis – methyl viologen treatment. In this assay seeds from lines homozygous for the transgene were used as starting material. The plants were grown *in vitro* at 30–50 μEinstein m^{-2} sec^{-1} for 2 weeks (16 h light and 8 h dark). The shoots were harvested in batches of 500 mg (roots were removed) and flooded in Petri dishes containing liquid medium with 10 μM methyl viologen and 0.1% Tween 20. The Petri dishes were incubated in the light (50 μEinstein m^{-2} sec^{-1}; 6 h light and 8 h dark) for 24 h. Before extraction the shoots were frozen at −70°C and subsequently thawed at 40°C. The chlorophyll was extracted with ethanol: 50 ml ethanol for 500 mg shoots. The OD of the extracts was measured at 663 nm.

Brassica napus – *heat and drought stress.* After pre-germination the seeds were transferred to 12 cm pots. After 10 days the seedlings were transferred to 45°C. Nine to 10 days later water was withheld for 3 days after which the plants were severely wilted. The plants were transferred to 23°C and watered normally. A few days later the damage to the plants was scored.

Whole mount TUNEL assay

Fixation was made with formalin acetic acid for 4 h. The samples were dehydrated with 50% (1 h) and 70% ethanol (1 h; refresh and store at −20°C). The tissues and cells were permeabilized with 0.3% Triton X100 (10 min) and 40 μg ml^{-1} proteinase K (15 min at room temperature). All the buffers contained 0.1% Tween 20 (Sigma, St Louis, MO, USA). DNA breaks were detected by using the '*In Situ* Detection Kit, Fluorescein' from Roche (Mannheim, Germany). For a

positive control fixed and permeabilized explants were treated for 1 h at 37°C with 40 U ml^{-1} DNaseI.

Labelling was evaluated by means of fluorescence microscopy (Axioplan 2; Zeiss, Jena, Germany). The nuclei that were labelled in the cortex of the explants were counted: 50 explants per line and condition.

ATP, total NAD^++NADH and superoxide content

ATP quantification was carried out as described (Rawyler *et al.*, 1999). Total NAD^++NADH was quantified as described (Filipovic *et al.*, 1999; Karp *et al.*, 1983). The formation of superoxides was quantified by measuring the reduction of 3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) (=XTT) as described (De Block and De Brouwer, 2002).

Poly(ADP-ribosyl)ation activity: immunological detection of poly(ADP-ribose)

For the isolation of plant nuclei and nuclear protein extraction the CelLytic PN kit of Sigma was used. Of each sample 0.5–1 μg of nuclear protein was spotted on a Hybond-C nitrocellulose membrane (Amersham, Buckinghamshire, UK). The poly(ADP-ribosyl)ated proteins were detected by means of anti-PAR antibodies (Calbiochem, San Diego, CA, USA) as primary antibody and anti-rabbit IgG alkaline phosphate conjugate (Sigma) as secondary antibody. Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI, USA) was used for staining. The image analysing software 'ImageJ 1.32j' (http://rsb.info.nih.gov/ij) was used to quantify the total intensity of the spots.

Oxygen consumption and TTC-reducing capacity

For measuring oxygen consumption a Clark polarographic electrode was used (Cyberscan DO310; Eutech Instruments, Singapore). The capacity of the plant lines to reduce 2,3,5-triphenyltetrazoliumchloride (=TTC) was quantified as described (De Block and De Brouwer, 2002).

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